CONVERSION OF 4-THIOURIDINE RESIDUES IN tRNA INTO RADIOACTIVE *N*-4-METHYLCYTIDINE FOR SEQUENCE ANALYSIS

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1. Introduction

Although sequence analysis of nucleic acids containing 4-thiouracil is hampered by the reactivity of the C:S group [1], this feature can be used to convert 4-thiouracil residues into stable *N*-4-methylcytidine residues labelled selectively with ^{14}C. This finding has already been briefly reported [2] and follows the discovery that cytidine is formed by the action of OsO_4 upon 4-thiouridine in the presence of NH_3 [3]. This article provides the essential details of the procedure and of its use in studying the sequence around the 4tU of $tRNA^{phe}$ from *Escherichia coli*. Independently, Ziff and Fresco [4] have applied a similar procedure with periodate as the oxidising agent to the study of valine tRNA. In this case, the sulphonate is known to be an intermediate [5]. Both methods are simple and adaptable to small-scale work. OsO_4 does not attack the *cis*-glycol at the 3′ terminus of the RNA and, under the conditions used, oxidation of ethylenic bonds of pyrimidines is very slight. The reaction with periodate is influenced by the conformation of the tRNA [4] but this factor is less important in the OsO_4 reaction.

2. Materials and methods

Unfractionated tRNA from *E. coli* strain CA244su⁻ was prepared at the Microbiological Research Establishment, Porton. The $tRNA^{phe}$ was prepared at Oak Ridge National Laboratory from *E. coli* B and was 65% pure [6]. Prostatic phosphomonoesterase and OsO_4 solutions were as described earlier [3]. RNAase T_1 was from the Sankyo Corp., spleen exonuclease from Worthington Biochemicals, and ^{14}C-methylamine from the Radiochemical Centre, Amersham. Nuclease digestions were performed according to Sanger and Brownlee [7] except that longer incubation times were required, probably because of the residual osmium in the modified RNA. The nucleosides were examined by paper chromatography in aq. 70% (v/v) propan-2-ol and in propan-2-ol/conc.HCl/water (13:4:2) by vol.

Abbreviations: tRNA, unfractionated transfer RNA; $tRNA^{phe}$, phenylalanine accepting tRNA; G,U,A,C, 4mC, 4tU, the 3′-phosphates of guanosine, uridine, adenine, cytidine, *N*-4-methylcytidine and 4-thiouridine respectively. In the figs. B is xylene cyanol F.F.used as a marker in ionophoresis and M is 4mC.

3. Conversion of 4-thiouridine into *N*-4methylcytidine

Previous reaction conditions for the formation of cytidine by OsO_4 and NH_3 were used [3] except that NH_3 was replaced by lower concentrations of methylamine hydrochloride buffers. The product was characterized by paper chromatography and UV spectrophotometry and yields were about 80% based on $\epsilon_{331} = 2.1 \times 10^4$ for 4-thiouridine [8,9]. Unprotonated CH_3NH_2 is like NH_3 in forming a complex with OsO_4 which is an active oxidising agent. Although 0.05 M CH_3NH_2 is oxidised by adding OsO_4, the rate appears to obey the equation $v = k[OsO_4]\,[CH_3NH_2]^3$. Hence this side reaction is not important in the reaction mixtures used which contained less than 10 mM OsO_4 and 5 mM unprotonated CH_3NH_2.

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3. Modification of 4-thiouridine residues in tRNA

In trial experiments, tRNA was treated with OsO_4 in aqueous ^{14}C-methylamine and the radioactivity was measured after precipitation by acid collection on membrane filters. The following conditions were eventually adopted: 1.0 mg of tRNA was dissolved in 70 μl of a solution containing 20 mM ^{14}C-methylamine (10 mCi/mmole) and 16 mM HCl. OsO_4 (10 μl of an aqueous 90 mM solution) was added. At this stage the pH was 8.0. After incubation for 1 hr at 37°C, residual osmium compounds were reduced by adding 10 μl of 2-mercaptoethanol and incubating for a further 10 min. The RNA was then precipitated by adding ethanol plus ammonium acetate, centrifuged and freeze dried.

Alkaline hydrolysis and paper ionophoresis at pH 3.5 showed a radioactive spot migrating just faster than C and identified as 4mC by treating with phosphomonoesterase and co-chromatography with nucleosides on paper. The amount of ^{14}C in the 4mC showed that approximately 1 mole of this nucleotide was formed per mole of tRNA. Although much additional ^{14}C was smeared along the ionophoresis papers, this labelling appeared to be spurious and was lost during two-dimensional ionophoresis on cellulose acetate at pH 3.5 and DEAE-paper in 7% formic acid [7]. The 4mC remained as the main spot just ahead of C in both dimensions and there was some ^{14}C at the origin,

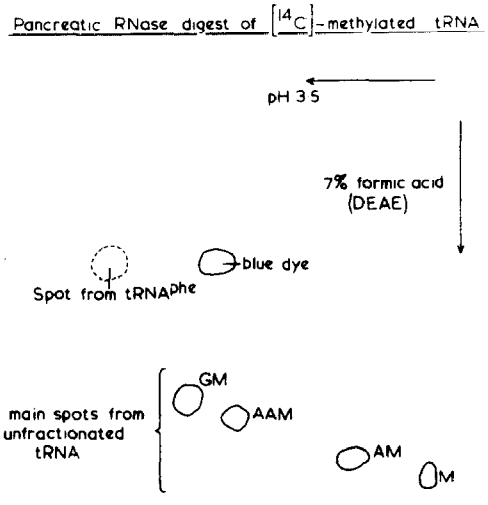

Fig. 1. Diagram showing positions of radioactive spots and the structures assigned to them in two-dimensional separations of pancreatic RNAase digests. M is *N*-4-methylcytidylic acid.

plus a faint spot near the position of A. The same pattern was given by tRNA^{phe}.

Digests of modified unfractionated tRNA with pancreatic RNAase A gave two main spots in positions expected for A4mC and G4mC with less intense 4mC and AA4mC spots (fig. 1). T_1-RNAase gave main spots in positions expected for 4mCG and (4mCA)G. Elution of the latter spot and treatment with pancreatic RNAase, showed that A4mCG was virtually absent.

One spot was given in each of the two types of nuclease digest of tRNA^{phe}. The structures were deduced by adding spleen exonuclease to the endonuclease digests and continuing the incubations for different periods of time before ionophoresis on DEAE-paper at pH 1.9 (fig. 3). From the relative mobilities of nucleotides in this system [7] and assuming that no unusual nucleotides are present apart from 4mC, the pancreatic RNAase product is GGA4mC and this is consistent with its position in fig. 1. On the same assump-

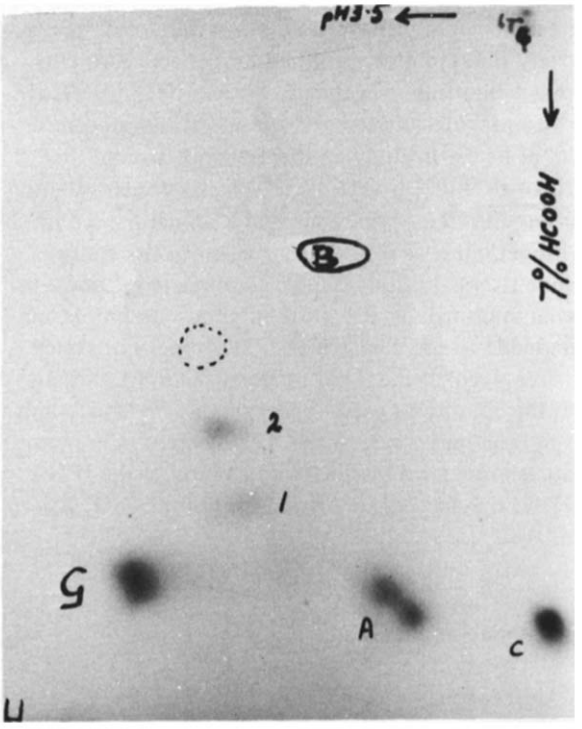

Fig. 2. Autoradiograph of two-dimensional separation of T_1 digest of modified tRNA. The broken circle shows the position of a minor spot which coincides on ionophoresis with the single spot from tRNA^{phe}. A,C,U and G, are marker 2′,3′-mononucleotides. B is the blue dye marker. Spot 1 is 4mCG and spot 2 is 4mCAG.

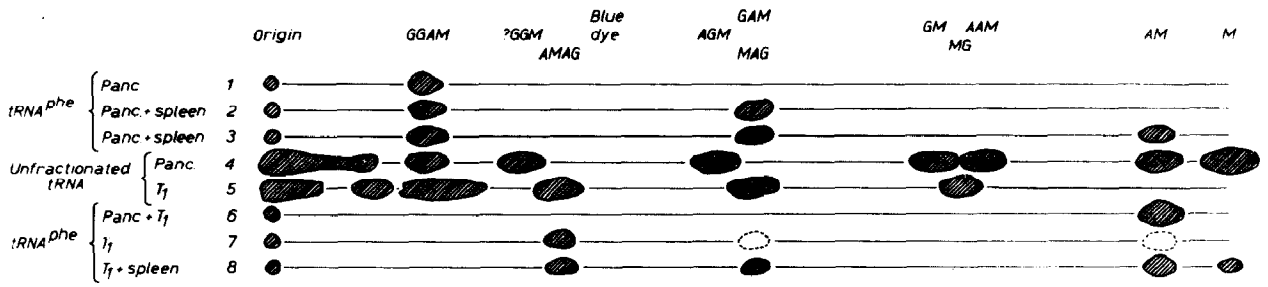

Fig. 3. Diagram of one-dimensional separation on DEAE-paper at pH 1.9. The broken circles in digest 7 indicate compounds released on digestion for 7 hr, apparently by a contaminating activity in the T_1-RNAase preparation. Enzymes used for digestion are indicated as follows: "Panc" = pancreatic ribonuclease. "T_1" = ribonuclease T_1. "spleen" = spleen exonuclease.

tion, the T_1 product is A4mCAG since, (i) its mobilities exclude extra C, A or U nucleotides, (ii) A4mC is released by pancreatic RNAase, (iii) no 4mCG was observed in any digest, (iv) a product with the mobility of 4mCAG is released by the T_1 and spleen enzymes acting together. (The appearance of the latter material on prolonged digestion with the T_1-preparation alone is presumably due to a contaminating enzyme). The two structures indicate an original sequence pyrimidine-GGA4tUAG which occurs in the complete structure given by Barrell and Sanger [1]. The results obtained in the present work are not compatible with the sequence CA4tUC(C)AG that has also been reported for the 4tU region of $tRNA^{phe}$ [10,11].

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References

[1] B.G.Barrel and F.Sanger, FEBS Letters 3 (1969) 275.
[2] K.Burton, Biochem. J. 114 (1969) 3OP.
[3] K.Burton, Biochem. J. 104 (1967) 686.
[4] E.B.Ziff and J.R.Fresco, Biochemistry 8 (1969) 3242.
[5] E.B.Ziff and J.R.Fresco, J. Am. Chem. Soc. 90 (1968) 7338.
[6] A.Dury, Science 157 (1967) 251.
[7] F.Sanger and G.G.Brownlee, Methods in Enzymology 12A (1967) 361.
[8] N.K.Kochetkov, E.I.Budowsky, V.N.Shibaev, G.I. Yeliseeva, M.A.Grachev and V.P.Demushkin, Tetrahedron 19 (1963) 1207.
[9] S.Irie, Y.Inoue and F.Egami, J. Biochem. (Tokyo) 63 (1968) 274.
[10] M.Uziel and H.G.Gassen, Biochemistry 8 (1969) 1643.
[11] H.G.Gassen and M.Uziel, Abstracts FEBS Madrid (1969) 128.